Activity of Galeterone in Hepatocellular Carcinoma and Description of Androgen Receptor Expression in Mouse Liver

Undergraduate Research Thesis

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ABSTRACT

Globally, liver cancer is the sixth most commonly diagnosed cancer, fourth leading cause of cancer deaths, and has increasing incidence and mortality rates [1]. Hepatocellular carcinoma (HCC) is the dominant form of liver cancer [1]. Although HCC treatments are improving, the 5-year survival rate in the United States remains low at 18% [2, 3]. A 3-fold higher prevalence of HCC in men than women suggests androgens, such as testosterone, promote the disease. Androgens bind and activate the androgen receptor (AR), a transcription factor that regulates a diverse set of genes. Despite evidence that AR contributes to HCC, traditional antiandrogen therapies, which are steroid-competitive at the ligand binding domain of AR, are ineffective in treating advanced HCC [4]. In primary HCC patient samples and HCC cell lines, we identified alternatively-spliced forms of AR (ARsvs) that lack the ligand binding domain, are constitutively active, and are unaffected by traditional antiandrogens (unpublished). We hypothesize that full-length AR and ARsvs contribute to liver disease progression, and novel selective AR degraders (SARDs) will have anti-HCC activity by inhibiting all forms of AR. The data support that pharmacologic activation or inhibition of full-length AR does not affect HCC cell line proliferation. However, a novel SARD, galeterone, decreases proliferation potentially by inhibiting full-length AR, ARsvs, and mTOR signaling. Future work aims to identify and investigate signaling through AR in immune and non-immune cells of the liver that is relevant to disease.

Key words: hepatocellular carcinoma, androgen receptor, antiandrogen.

INTRODUCTION

Cancer is projected to have affected 17 million people and caused 9.6 million deaths in 2018 worldwide [1]. Globally, liver cancer is the sixth most commonly diagnosed cancer, fourth leading cause of cancer deaths, and has increasing incidence and mortality rates [1]. The dominant form of liver cancer is hepatocellular carcinoma (HCC) [1]. Due to the disease and risk factors presenting few early symptoms, HCC patients are often diagnosed at an advanced stage [5-7]. Although treatment options are improving, the 5-year survival rate for patients diagnosed at an advanced stage, which includes over half of the patient population, remains low at 18% in the United States [2, 3].

Interestingly, HCC is a sexually dimorphic disease with 3- to 4-fold higher prevalence in men than women depending on the region [1]. Because prevalence of HCC is higher in males than females, it is thought that androgens, such as testosterone, promote HCC [8]. Androgens bind and activate the androgen receptor (AR). Once activated, AR can enter the cell nucleus, where it functions as a transcription factor to regulate androgen-responsive gene expression.

Despite evidence for AR expression in primary HCC tumors and AR activity promoting HCC, traditional antiandrogen therapies that antagonize AR at the ligand binding domain are ineffective in treating advanced HCC [4]. However, the antiandrogen therapies previously tested in HCC were unable to bind or inhibit alternatively-spliced, C-terminal truncated forms of AR (ARsvs), which are constitutively active and known to confer resistance to antiandrogen therapies that antagonize AR at the C-terminal ligand binding domain in prostate cancer [9, 10]. We recently identified expression of ARsvs in primary HCC patient samples and HCC cell lines. Based on the expression of ARsvs in HCC and their activity in prostate cancer, we hypothesize that full-length AR and ARsvs contribute to liver disease progression. Moreover, novel selective AR degraders (SARDs) that inhibit all forms of AR are expected to have anti-HCC activity. This hypothesis was investigated by testing the effects of the AR antagonist

enzalutamide, which binds to the ligand-binding domain, and the SARD galeterone in HCC cell lines. We found that the AR antagonist enzalutamide does not decrease HCC proliferation; however, galeterone reduced proliferation, potentially by attenuating signaling of mTOR, full-length AR, and ARsv signaling. Galeterone and similar SARDs may be a therapy option for AR-positive HCC tumors, which are estimated to comprise approximately 2/3 of primary HCC tumors [11-13]. To further understand AR signaling and efficacy of SARDs, CRIPSR/Cas9-mediated knockout of total AR or only ARsvs will be used to study the relative contribution of ARsvs in HCC and the activity of SARDs like galeterone against ARsvs.

Although AR-positive HCC tumors comprise the majority of HCC [11-13], preliminary findings in our lab suggest that the proportion of AR-positive hepatocytes in healthy mouse and rat liver is low, and in cells expressing AR, the expression is low. The finding that AR expression is low in hepatocytes led us to investigate cells other than hepatocytes, including intrahepatic immune cells, to locate cells in which AR signaling relevant to disease may occur. Elucidating AR and immune cell signaling satisfies a need to understand potentially sexually dimorphic immune cell responses to liver disease, a contribution that can inform development of novel therapies, including immune-modulating therapies recently introduced to HCC [2].

Based on higher prevalence of HCC in men than women, we hypothesize that AR signaling in immune and non-immune cells of the liver promotes an environment that favors liver disease, including HCC. Using flow cytometry, AR expression in immune and non-immune cells of the liver was investigated, and the results demonstrated a subset of each immune and non-immune cells that express AR at a low level. The molecular and macrocellular consequences of AR signaling in these subsets and the effects of hormone signaling to immune cell action in the liver will be further studied using *in vivo* models of liver disease and cancer.

MATERIALS AND METHODS

Cell Culture

HCC cell lines SNU423, HepG2, LM3, and the prostate cancer cell line LNCaP were obtained from American Type Culture Collection (ATCC) and incubated at 37°C in a humidified chamber containing 5% CO_2. LNCaP and SNU423 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). LM3 cells were cultured in DMEM (Gibco) supplemented with 10% FBS. HepG2 cells were cultured in MEM (Gibco) supplemented with 10% FBS.

Animals

Female C57BL/6J mice were purchased from the Jackson Laboratory and kept at the Research Animal Facility of The Ohio State University. The experimental procedures and protocols used in this study were approved by the Animal Care and Use Committee of the Ohio State University.

Proliferation assay

Cells were plated in a 96-well plate at 800 cells/well for SNU423, 1,200 cell/well for LM3, 3,000 cells/well for HepG2, and 5,000 cell/well for LNCaP in complete medium supplemented with 10% FBS and incubated overnight. Medium was refreshed the following day with phenol-free medium supplemented with 5% charcoal/dextran-treated FBS, and cells were incubated for 24 hours. Medium was then refreshed with phenol-free medium supplemented with 5% charcoal/dextran-treated FBS and 1 nM AR agonist R1881 or ethanol as vehicle treatment as 0.1 % of the final experimental volume. After incubating for 72 hours, CCK-8 (Dojindo Molecular Technologies) was added to the culture medium. Cells were incubated for 2 hours in the dark at 37°C in a humidified chamber containing 5% CO_2 followed by measuring absorbance at 450nm.

Western Blot

Cells were plated in a 6-well plate at 260,000 cells/well in complete medium supplemented with 10% FBS and incubated overnight. Medium was refreshed with phenol-free medium supplemented with 5% charcoal/dextran-treated FBS and cells were incubated for 24 hours. Medium was then refreshed with phenol-free medium supplemented with 5% charcoal/dextran-treated FBS and the indicated drug or ethanol only as the vehicle solution as 0.1% of the final experimental volume. Protein lysates were collected with RIPA lysis buffer supplemented with 1% Protease/Phosphatase Inhibitor Cocktail (Thermo Scientific).

Protein concentration was determined by Bradford assay, and 30 µg of protein lysate was resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein was transferred to a nitrocellulose membrane followed by incubating in the following primary antibodies: amino-terminus directed anti-AR (1:2000, Cell Signaling, Cat# D6F11), anti-phospho p70S6K (Thr389; 1:1000, Cell signaling, Cat# 108D2), anti-p70S6K (1:1000, Cell Signaling, Cat# 49D7) anti-phospho-4EBP1 (Thr37/46; 1:2000, Cell Signaling, Cat# 236B4), anti-4EBP1 (1:2000, Cell Signaling, Cat# 53H11), and anti-GAPDH (1:5000, Cell Signaling, Cat# D16H11). Following primary antibody incubation, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody and imaged with Pierce ECL chemiluminescent substrate (Thermo Scientific) for all targets except AR, which was imaged with SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Scientific).

Isolation of Hepatic Cells

Fresh mouse liver was processed with mouse liver dissociation kit, gentleMACS C tubes, and gentleMACS Octo Dissociator (Miltenyi Biotec) to obtain a single cell suspension. Mouse liver was removed and washed once with cold DMEM (Gibco). The liver was transferred to a gentleMACS C tube

containing the provided digestion enzymes and processed for 28 seconds by the gentleMACS Octo Dissociator followed by incubating at 37°C in a humidified chamber containing 5% CO_2 for 30 minutes on an orbital shaker. The resulting suspension was then processed for 14 seconds by the gentleMACS Octo Dissociator followed by filtering through a 70-µm cell strainer. Samples were centrifuged and resuspended in 1 ml cold PBS. A volume of 9 ml of 1X RBC lysis buffer (eBioscience) was added to the suspension and incubated for 6 minutes at room temperature followed by a second strain through a 70-µm cell strainer. All samples were washed with cold PBS, and approximately one million cells per panel were aliquoted for antibody staining.

Flow Cytometry

Cells were incubated with LIVE/DEAD Fixable Near-IR Dead Cell Stain (Invitrogen) diluted 1:333 in PBS for 15 minutes at room temperature in the dark. Surface antigen staining was performed at 4°C for 30 minutes in the dark with APC anti-mouse CD45 (1:200, Biolegend, Clone 30-F11).

Intracellular staining of AR was performed with the Foxp3 Fixation/Permeabilization kit (Invitrogen) according to the manufacturer's protocol and incubating with PE amino-terminus directed anti-AR (1:50, Santa Cruz, Cat# sc-7305) for 60 minutes at room temperature. The BD LSR Fortessa (BD Biosciences) was used for data acquisition, and data was analyzed by FlowJo software (FlowJo, LLC).

CRISPR/Cas9

Alt-R CRISPR-Cas9 crRNA (Integrated DNA Technologies, IDT) and Alt-R CRISPR-Cas9 tracrRNA (IDT) were combined in Nuclease-Free Duplex Buffer (IDT) at 1 µM final concentration each. The Alt-R CRISPR-Cas9 crRNA sequences are listed in Table 1. The solution was heated at 95ºC for 5 minutes

and placed at room temperature until cooled to 25ºC to form gRNA oligos comprised of the annealed crRNA and tracrRNA.

LNCaP cells plated at 15,000 cells/well and SNU423 cells plated at 6,000 cell/well were plated the day prior to transfection in complete medium. The transfection complex was added to each well as described below. A ribonucleoprotein (RNP) complex of Alt-R S.p. HiFi Cas9 Nuclease V3 (IDT) and gRNA was created by incubating a solution of gRNA (60 nM), Cas9 enzyme (60 nM), and Cas9 PLUS Reagent (2.4% v/v% from Invitrogen) diluted in Opti-MEM reduced serum medium (Gibco) for 5 minutes at room temperature. A volume of 12.5 µl of each of two RNP complexes were combined with 1.2 µl Lipofectamine CRISPRMAX Cas9 Transfection Reagent (Invitrogen) and Opti-MEM reduced serum medium to 50 µl final volume and incubated 20 minutes at room temperature to form the transfection complex.

A volume of 50 µl of the transfection complex was added to each well of a 96-well plate containing 100 µl of medium. After incubating for 48 hours, DNA was collected by washing each well with PBS and adding 15 µl QuickExtract DNA Extraction Solution (Lucigen). The lysate was collected and heated at 65°C for 10 minutes followed by 98°C for 5 minutes.

Table 1. crRNA Sequences

crRNA ID	Sequence
1	CAC ATT AGG GAA CTT CTT GT
2	TAG CCT TTC AAT CCA CAT TA
3	GCA CAT CCT TGC CAA AGC AC
4	TGT TGG ACT ATT TAG TCT AA

Polymerase Chain Reaction (PCR)

PCR was performed with a reaction of Q5 High-Fidelity 2X Master Mix (New England Biolabs), 30 ng DNA from transfected cells, 500 nM forward primer (GCA GGG TGA GGA TGG TTC T), and 500

nM reverse primer (ATG CTC CAA CGC CTC CA). The resulting amplicon was resolved on an agarose gel containing ethidium bromide.

Statistical Analyses

Data analyses and statistical tests were performed in GraphPad Prism version 8.00 for Windows (GraphPad Software) using the statistical tests as indicated in figure legends.

RESULTS

Ligand-Dependent AR Activity Does not Increase Proliferation in HCC Cell Lines

HCC cell lines with AR expression (SNU423 and LM3) or without AR expression (HepG2) and an AR-positive prostate cancer cell line (LNCaP) as a positive control were treated with the AR agonist R1881 in hormone-deplete medium, and proliferation was measured.

The data demonstrated that activation of only full-length AR by R1881 did not increase proliferation relative to vehicle treatment regardless of AR expression level in HCC cell lines (Figure 1). However, as expected, the prostate cancer cell line showed increased proliferation in response to activating AR. This data supports that unlike prostate cancer, the genes regulated by full-length AR in HCC are not likely to contribute to proliferation.

As a potential explanation for these data, it is possible that the genes contributing to proliferation in HCC are regulated in a receptor-dependent but ligand-independent manner by ARsvs, and activation of full-length AR by ligand does not further promote proliferation. To further address the possibility of receptor-dependent but ligand-independent regulation of proliferation by ARsvs in HCC, the effect to proliferation resulting from degradation of full-length AR and ARsvs by the SARD galeterone or antagonism of full-length AR by enzalutamide was evaluated.

Galeterone Decreases HCC Proliferation

Given the evidence that activating full-length AR did not increase proliferation of HCC cell lines, we hypothesized that traditional antiandrogens that inhibit only full-length AR, such as enzalutamide, would not decrease proliferation in HCC cell lines. However, the SARD galeterone was expected to reduce proliferation by inhibiting constitutive, ligand-independent ARsv signaling. To test this hypothesis, HCC

cell line proliferation was measured following treatment with enzalutamide or the SARD galeterone in hormone-deplete medium with or without addition of the AR agonist R1881.

As expected, enzalutamide did not decrease HCC proliferation in the presence or absence of the AR agonist (Figure 2). However, galeterone did reduce proliferation of HCC cell lines in the presence or absence of the AR agonist, and this effect was most potent in AR-positive HCC cell lines SNU423 and LM3 (Figure 2). Interestingly, galeterone's anti-proliferative effects were most potent in the cell line with the greatest expression of ARsvs, LM3 (Figure 2). ARsvs are constitutively active and functional in both the presence and absence of the AR agonist, and because proliferation was decreased by galeterone to the same extent with or without the AR agonist, galeterone's action to decrease proliferation in HCC cell lines may in part be explained by inhibition of ARsvs, which may play a role in HCC proliferation. However, galeterone also decreases proliferation in the AR-negative HCC cell line HepG2 although at greatly reduced potency compared to AR-positive cell lines SNU423 and LM3 (Figure 2).

An effect of galeterone in the AR-negative cell line suggests that the drug targets proteins other than AR to exert its effects in HCC cell lines. Moreover, inhibiting the other potential targets of galeterone may not affect prostate cancer cell line proliferation. In the prostate cancer cell line LNCaP, galeterone and enzalutamide had an effect only in the presence the AR agonist, suggesting that galeterone reduces LNCaP proliferation predominately by blocking full-length AR activity. Alternatively, ligand-independent ARsv signaling may not meaningfully contribute to LNCaP proliferation. Due to degrading full-length AR and ARsvs as well as potentially modulating other pathways that regulate HCC proliferation, drugs like galeterone may be capable of treating AR-positive HCC tumors, which are estimated to comprise about 2/3 of primary HCC tumors [11-13].

Galeterone Decreases Proliferation in part by Degrading AR and Inhibiting mTOR Signaling

Based on work by Zhang et. al. demonstrating that inhibiting both AR and mTOR has greater anti-HCC activity than inhibiting only AR or mTOR [14], we hypothesized that galeterone's activity in HCC may be due to dual-inhibition of AR and the mTOR pathway. Indeed, galeterone has been shown to inhibit signaling through the mTOR pathway, including Mnk-eIF4E signaling, to decrease growth and motility of *in vitro* and *in vivo* models of pancreatic [15] and prostate cancer [16]. To test whether mTOR inhibition is involved in galeterone's activity in HCC, SNU423 and LM3 HCC cell lines were treated with several concentrations of galeterone or enzalutamide, and AR protein levels and activation of the mTOR pathway were measured by Western blot.

As expected, enzalutamide, an AR antagonist, did not alter the protein level of AR nor decrease activation of the mTOR pathway based on levels of phosphorylated Akt and S6K, downstream targets of mTOR, in either cell line (Figure 3). Galeterone decreased AR protein in both cell lines in a concentration-dependent manner. The effects of galeterone on the mTOR pathway varied with concentration and cell line. In SNU423, galeterone decreased both phosphorylated Akt and S6K at 1 μM, yet as the concentration of galeterone increased, the phosphorylation of Akt and S6K generally increased. In LM3 cells, galeterone caused an increase in phosphorylated Akt with increasing concentrations of drug, yet phosphorylated S6K was decreased at all concentrations. These results support that in general, degradation of AR is in a direct relationship with the concentration of galeterone. However, galeterone's effects on the mTOR pathway suggest that galeterone inhibits the mTOR pathway at lower concentrations near approximately 1 μM yet results in mTOR pathway activation as concentrations increase above approximately 1 μM.

We hypothesize that the effects on the mTOR pathway by galeterone are due to nonspecific proteasomal degradation of proteins other than AR at high concentrations of galeterone, thereby necessitating a need for increased mTOR pathway activation to transcribe mRNA. To test whether mTOR pathway activation occurs as result of nonspecific galeterone-mediated proteasomal protein degradation,

cells could be co-treated with increasing concentrations of galeterone in the absence or presence of a proteasomal inhibitor, such as MG132. Evidence that the mTOR pathway is not activated at higher concentrations of galeterone when a proteasomal inhibitor is introduced would suggest that activation of the mTOR pathway is in part caused by galeterone-mediated protein degradation. Regardless of the unclear effects of galeterone on the mTOR pathway, the drug inhibits full-length AR, ARsv, and mTOR signaling at certain concentrations. Due to this activity, galeterone and similar drugs may have applications to HCC therapy as a relatively improved therapy compared to the traditional antiandrogens, which antagonize only full-length AR.

Analysis of AR Protein Levels in Immune and Non-Immune Cells of the Liver

While evaluating galeterone and other novel antiandrogens in HCC cell lines provides valuable insight into the molecular mechanism of action within an HCC tumor, *in vitro* experiments do not account for the complexity of an intact liver. The liver is comprised of both immune and non-immune cells. Following a carcinogenic, toxic, or viral challenge, immune cells are recruited to the liver to aid in repair and clearance of damaged cells. Interestingly, there appears to be a sexually dimorphic immune cell response to liver damage that is at least partially explained by signaling through AR [17]. However, the sexually dimorphic signaling relevant to liver disease, cancer onset and progression, or antiandrogen treatment remains unclear. Moreover, the cells, including a broad immune and non-immune cell distinction and specific cell subsets of each, in which AR signaling relevant to disease occurs is not understood.

To provide insight into the distribution of cell that express AR in the liver, AR expression in both immune (CD45+) and non-immune cells (CD45-) was measured by flow cytometry. AR is expressed in both immune (CD45+) and non-immune (CD45-) cells of the liver although at low levels in both populations (Figure 4). Although these preliminary data are from healthy mice, the distribution and level

of AR expression in the liver raises important questions regarding the influence of hormone therapies, including SARDs like galeterone, on immune cell signaling and recruitment at the liver. Additionally, this hormone signaling may be relevant to immune-modulating therapies that have recently been introduced for treatment of HCC. For instance, if AR signaling in a cancerous liver or a HCC tumor prevents the healthy immune response to target pre-cancerous and cancerous cells, a dual-therapy with antiandrogen and an immune-modulating agent may provide improved outcomes relative to a monotherapy of either alone.

CRISPR/Cas9-mediated Knockout of total AR or only ARsvs

To better understand the contribution of ARsvs to the action of galeterone, as well as other SARDs, to the HCC onset or progression in *in vitro* and *in vivo* models of HCC, CRISPR/Cas9 was used to knockout expression of total AR or only ARsvs. Recent work by Etten et. al. demonstrated that obstructing a polyadenylation site in intron three of the AR gene with a morpholino prevented expression of ARsvs that utilize cryptic exon three, yet full-length AR expression was not affected by the morpholino [20]. Based on the findings of Etten et. al., we hypothesized that excising the polyadenylation site in intron three will selectively knockout biologically relevant ARsvs. Using CRISPR/Cas9 to target an early exon of the AR gene, total AR-knockout cells will also be generated.

A 1000 bp region of DNA encompassing the polyadenylation site in the AR gene that Etten et. al. demonstrated to be essential for ARsv expression was PCR amplified from cells transfected with Cas9 enzyme and gRNA targeting the AR gene or control gRNA. If Cas9 editing is successful, there is expected to be an approximately 500 bp band on the agarose gel that represents Cas9-mediated excision of a 500bp region of DNA that encompasses the polyadenylation site in intron three of the AR gene. The data demonstrate that a 500 bp band was produced with gRNA combinations 1+4 and 2+3 in LNCaP cells and

2+3 in SNU423 cells. These data support that the CRISPR/Cas9 strategy was successful at the genomic level in a fraction of the population of cells (Figure 5).

Future work includes clonally expanding edited cells by limiting dilution and verifying that excision of the polyadenylation site in intron three of the AR gene prevents ARsv mRNA and protein expression in these edited cells. Additionally, the efficiency of the gRNAs that guide Cas9 to an early exon of the AR gene for total AR knockout will be estimated by Inference of CRISPR Edits software [18]. Following clonal expansion of edited cells, total AR knockout will be verified by mRNA and protein analysis.

The resulting total AR and ARsv-specific knockout cell lines created by CRISPR/Cas9 will be a valuable tool to study the relative contribution of ARsvs to cellular functions and the effects of antiandrogens on ARsvs. These edited cell lines will also permit study of full-length and splice variant AR to cancer progression *in vivo* by using the cell lines to create orthotopic tumors.

DISCUSSION

In HCC cell lines, activating full-length AR with ligand R1881 did not increase proliferation (Figure 1). However, degradation of full-length AR and ARsvs by galeterone decreased proliferation in the presence or absence of AR ligand (Figure 2). Moreover, galeterone showed activity in an AR-negative HCC cell line (HepG2) and was most potent in the HCC cell line with the greatest ARsv activity (LM3). Based on this evidence, galeterone likely acts on proteins other than full-length AR, including ARsvs and the mTOR pathway, to decrease HCC cell line proliferation. However, due to the broad effects and expansive inputs and outputs of AR signaling and the mTOR pathway, it is expected that these postulated targets are only one of many pathways altered by galeterone at anti-proliferative concentrations. Nonetheless, dual-inhibition of both AR and mTOR by galeterone and similar drugs may represent a therapeutic strategy to treat AR-positive HCC.

To understand the contribution of ARsvs to the action of galeterone, as well as other SARDs, to HCC onset or progression in *in vitro* and *in vivo* models, CRISPR/Cas9 will be used to knockout expression of total AR by creating a missense mutation or of only ARsvs by excising a polyadenylation site in intron three of the AR gene. Total AR and ARsv-specific knockout cell lines created by CRISPR/Cas9 will prove as valuable tools to study the relative contribution of full length-AR and ARsvs to the biology of HCC and liver disease by using these cell lines in *in vivo* orthotopic cancer models, for example. Evidence that tumors expressing both full-length AR and ARsvs grow more quickly or are more invasive than ARsv-knockout derived tumors would support investigation of novel SARDs like galeterone in treatment of HCC due to the SARDs' ability to inhibit all forms of AR.

To understand the overall liver environment that leads to cancer, flow cytometric analysis will be used to study recruitment and signaling, including AR signaling, in intrahepatic immune cells. Our preliminary flow cytometry findings support that AR is expressed in immune and non-immune cells of the liver. Based

on our observed pattern of AR expression, we hypothesize that AR signaling in both immune and non-immune cells contributes to sexual-dimorphism of liver diseases. Future work aims to test this hypothesis by using flow cytometric and immunohistochemical analysis of AR expression in specific immune and non-immune subsets of liver cells to identify AR-expressing populations in healthy and damaged liver. Additionally, by isolating cell subsets by fluorescence activated cell sorting, potential differences in gene expression for cell subsets in healthy and diseased liver can be investigated. Due to sexual dimorphism of liver cancer, AR signaling in immune cells is expected to promote a gene expression pattern that contributes to a chronically inflamed environment favoring onset or progression of liver disease. In addition to AR, estrogen receptor (ER) signaling in immune and non-immune cells of the liver will be investigated in future experiments. ER signaling may act protectively in the liver and contribute to the observed sexual dimorphism liver disease prevalence.

CONCLUSION

In HCC cell lines, activating full-length AR did not increase proliferation. Enzalutamide did not affect proliferation of HCC cell lines, which was expected since enzalutamide inhibits only full-length AR and drugs that inhibit only full-length AR fail to treat advanced HCC. Galeterone, a novel SARD, decreased proliferation at least in part by inhibiting full-length AR, ARsv, and mTOR pathway signaling. Future work aims to study ARsv signaling, which we have detected in primary HCC patient samples and HCC cell lines (unpublished), by using CRISPR/Cas9 to knockout total AR or specifically ARsvs. Using flow cytometry, AR expression was detected in both immune and non-immune cells of the liver, supporting analysis of AR signaling in immune cells in healthy and diseased liver. Insight into the influence of full-length AR, ARsv, and broader hormone signaling in immune and non-immune cells of the liver may provide insight into the sexual dimorphism of HCC and inform development of improved treatments.

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FIGURES

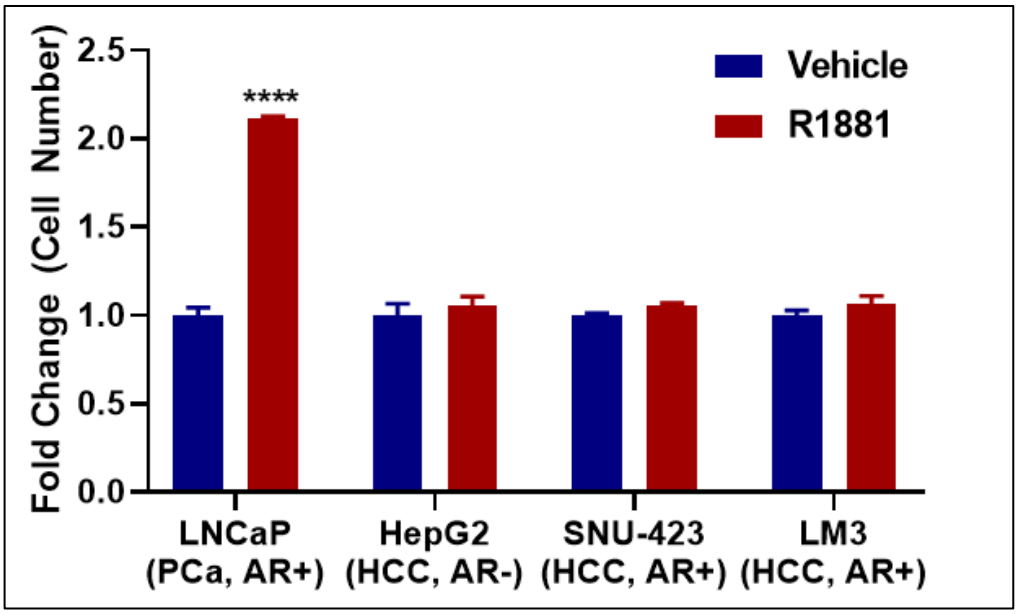

Figure 1. Activating Full-Length AR in HCC Cell Lines Does Not Increase Proliferation.

The prostate cancer cell line LNCaP (AR+) and HCC cell lines HepG2 (AR-), SNU-423 (AR+), and LM3 (AR+) were cultured in phenol-free medium supplemented 5% charcoal stripped serum with or without 1 nM R1881 for 72 hours. CCK-8 reagent was used to measure cell number. (****, $p<0.0001$ by Sidak's multiple comparison test).

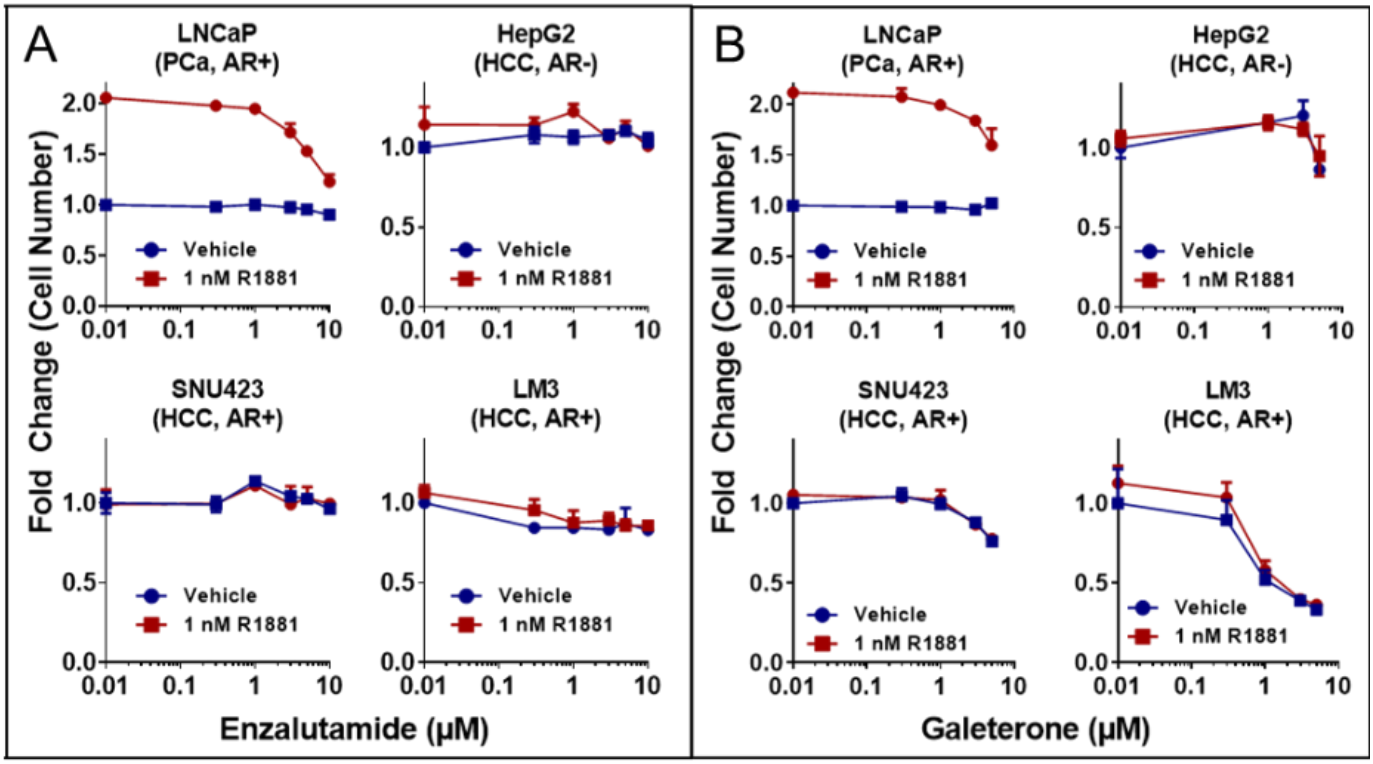

Figure 2. Galeterone Decreases AR-Positive HCC Cell Line Proliferation.

The prostate cancer cell line LNCaP (AR+) and HCC cell lines HepG2 (AR-), SNU-423 (AR+), and LM3 (AR+) were cultured in phenol-free medium supplemented 5% charcoal stripped serum. Cells were treated with increasing concentrations of enzalutamide (A) or galeterone (B) in the presence or absence of 1 nM R1881 for 72 hours. CCK-8 reagent was used to measure cell number.

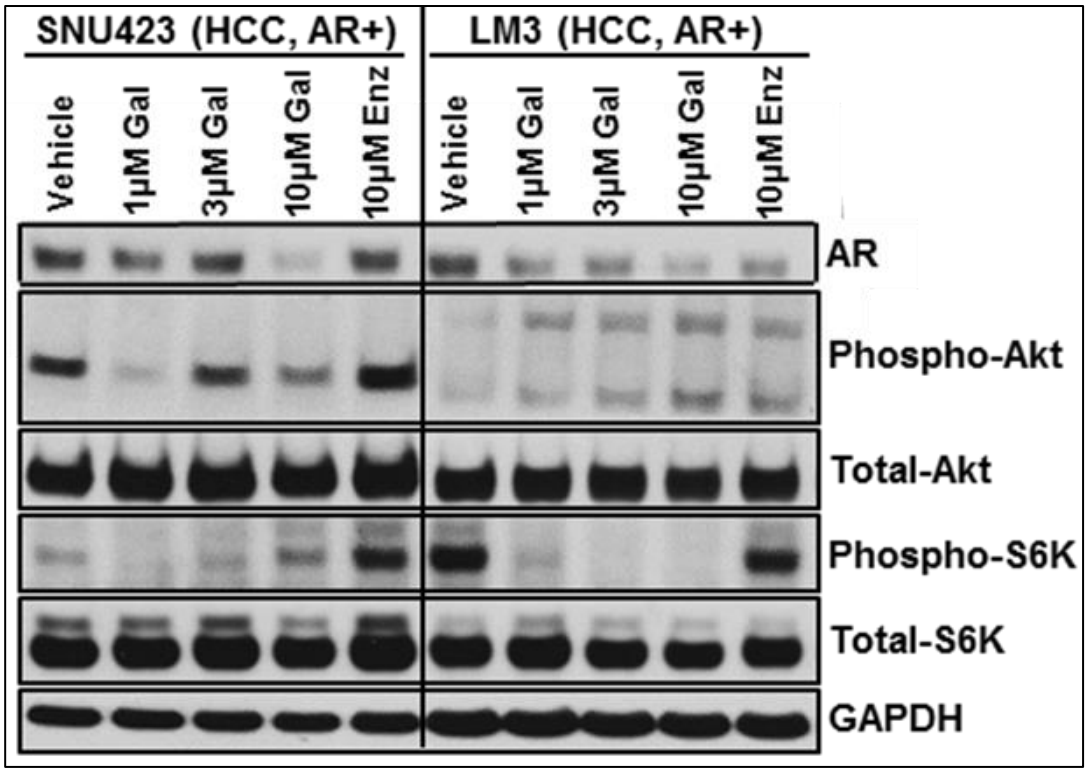

Figure 3. Galeterone Decreases AR Protein and mTOR Pathway Activation.

SNU-423 and LM3 HCC cell lines were cultured in phenol-free medium supplemented 5% charcoal stripped serum and treated with enzalutamide or galeterone at the indicated concentration for 24 hours. Protein levels of AR, phosphorylated and total Akt, phosphorylated and total S6K, and GAPDH were measured by Western blot.

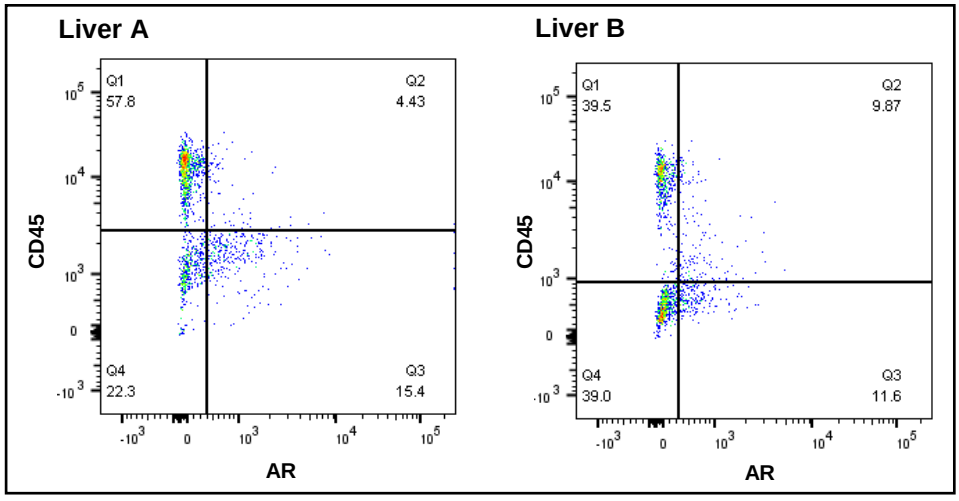

Figure 4. AR is Expressed in Immune (CD45+) and Non-Immune (CD45-) Cells of the Liver.

Representative dot plots from flow cytometry analysis of AR expression in immune (CD45+) and non-immune (CD45-) cells from two adult, healthy mouse livers.

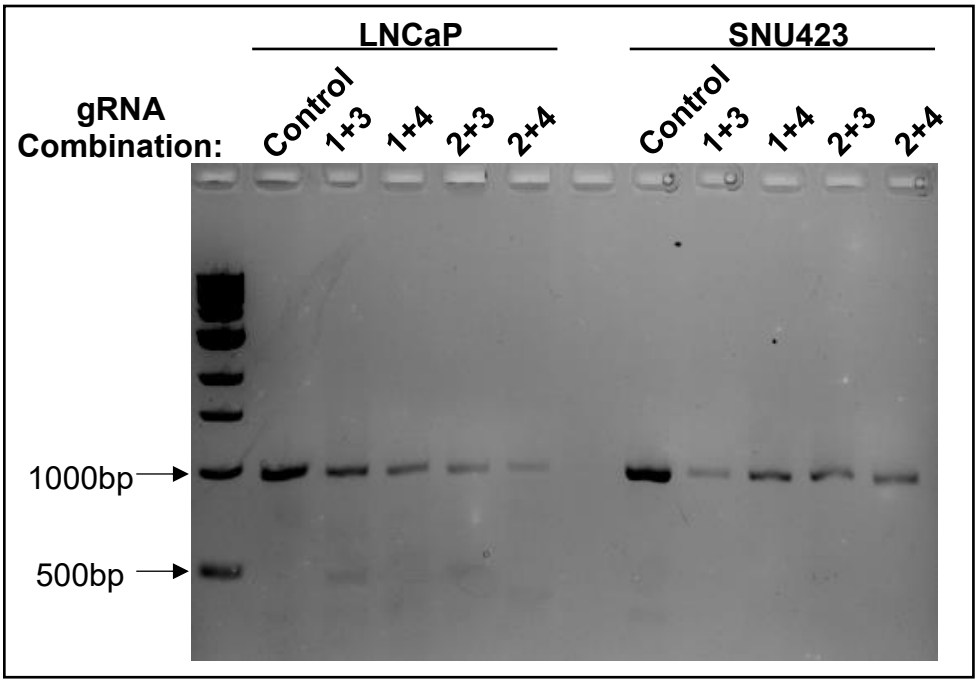

Figure 5. CRISPR/Cas9-Mediated Excision of a Region Encompassing a Polyadenylation Site in Intron 3 of the AR Gene.

Resulting PCR product of DNA from LNCaP and SNU423 cells transfected with the indicated combination of gRNA or control gRNA (gRNA targeted to the HPRT gene, not AR). The 500 bp band is representative of successful Cas9-mediated excision of a 500 bp region encompassing a polyadenylation site in intron three of the AR gene.